# DELIGNIFICATION OF BANANA STEM WASTE IN ANAEROBIC CONDITIONS BY USING *Bacillus cereus*

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I declare that this thesis entitled "*Delignification of Banana Stem Waste in Anaerobic Conditions by Using Bacillus cereus*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not currently submitted in candidature of any other degree.

Signature : …….

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Date : …….

To my dearly loved mother, Robitah binti Hijaz,
father, Baharuddin bin Abd Rahim,
and sisters

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# ABSTRACT

Today, the productions of biodegradable plastics are crucial due to the environment problems that keep escalating caused by conventional plastics. Many efforts have been done to reduce the cost of biodegradable plastic including by using waste as raw material. Production of biodegradable plastics by microorganism needs carbon sources. Several ranges of microorganisms have been tested for their ability to degrade lignin in order to obtain cellulose content to be used as carbon source. In this study, the submerged fermentation in anaerobic conditions of banana stem waste using *Bacillus cereus* and the capability of *Bacillus cereus* to delignify banana stem waste in anaerobic conditions are investigated. This study was divided into three parts which are the revival of *Bacillus cereus*, growth curve determination of *Bacillus cereus*, and delignification fermentation and analysis of *Bacillus cereus*. Fermentation was carried out anaerobically in batch mode at 30 $^{o}$C and 37 $^{o}$C. The analysis was done in accordance to Klason method. Results showed that *Bacillus cereus* was able to survive for 48 hours in anaerobic conditions and it is able to degrade lignin from banana stem waste. The optimum temperature for *Bacillus cereus* to degrade lignin was at 30 $^{o}$C with average percentage of lignin degradation of 13.33 %.

# ABSTRAK

Dewasa ini, penghasilan plastik terbiodegradasikan menjadi amat penting memandangkan masalah alam sekitar yang kian meningkat disebabkan oleh plastik biasa. Banyak usaha telah dilakukan bagi mengurangkan kos penghasilan plastik terbiodegradasikan ini termasuk penggunaan sisa sebagai bahan mentah. Penghasilan plastik terbiodegradasikan ini memerlukan sumber karbon sebagai nutrien bagi mikroorganisma. Beberapa jenis mikroorganisma telah diuji kebolehannya untuk mengurangkan lignin dalam sisa tumbuhan bagi mendapatkan kandungan selulosa di dalamnya untuk digunakan sebagai sumber karbon. Dalam kajian ini, kaedah fermentasi terendam batang pisang dalam keadaan anaerobik menggunakan *Bacillus cereus* dan kebolehan *Bacillus cereus* untuk mengurangkan lignin dalam batang pisang telah dikaji. Kajian ini dibahagikan kepada tiga bahagian iaitu menghidupkan semula *Bacillus cereus*, penentuan graf pertumbuhan bagi *Bacillus cereus*, dan proses fermentasi dan analisis bagi pengurangan lignin oleh *Bacillus cereus*. Proses fermentasi telah dijalankan secara anaerobik pada suhu 30 $^{o}$C and 37 $^{o}$C. Manakala proses analisis telah dijalankan mengikut kaedah Klason. Hasil kajian menunjukkan bahawa *Bacillus cereus* berupaya untuk hidup selama 48 jam dalam keadaan anaerobik. Ia juga terbukti boleh mngurangkan lignin dalam batang pisang. Suhu optimum bagi *Bacillus cereus* untuk mengurangkan lignin dalam keadaan anaerobik ialah pada 30 $^{o}$C dengan peratusan purata sebanyak 13.33 %.

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## LIST OF SYMBOLS/ABBREVIATIONS

| | | |
|---|---|---|
| % | - | Percentage |
| $^{o}C$ | - | Degree Celcius |
| g | - | Gram |
| kg | - | Kilogram |
| L | - | Liter |
| $MgSO_4.7H_2O$ | - | Hydrated magnesium sulphate |
| mL | - | Mililiter |
| mm | - | Milimeter |
| rpm | - | Rotation per minute |
| sp. | - | Species |
| v/v | - | Volume per volume |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

In the recent years, lack of degradability, the closing of landfill sites, and the growing of water and land pollution problems have led to concern about plastics. Conventional plastics that are synthetically derived from petroleum are not readily biodegradable (Huang *et al*., 1991; Young, 1981) and are considered as environmentally harmful wastes (Hong Kong Environmental Protection Department, 1994). Plastic materials account for about 20% by volume of municipal solid wastes and reduce the capacity of precious landfill sites (Lee and Yu, 1997). This is why there are growing interests in the production of bioplastics. Environmentally friendly biodegradable plastic, or simply bioplastic, have been developed either by incorporating natural polymers into conventional plastic formulations, by chemical synthesis, or by microbial fermentations (Chua *et al*., 1995; Chang, 1994). Despite receiving much attention as a biodegradable substitute for conventional non-biodegradable plastics, the commercial use of bioplastics remains limited because of its high production cost (Jung *et al*., 2005).

A family of more than 40 poly-hydroxy alkanoates (PHAs) and their co-polymeric derivatives are considered as very attractive materials in producing bioplastic due to their complete biodegradability (Kumagai, 1992). They are polymers produced by microorganisms as inclusion bodies, susceptible to biodegradation into carbon dioxide and water (Kapritchkoff *et al*., 2006). Poly-β-hydroxybutyric acid (PHB) is the

most extensively studied among the poly-hydroxy alkanoates in nature in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power (Kapritchkoff *et al*., 2006; Salehizadeh and Van Loosdrecht, 2004; Pfeffer, 1992). PHB has properties similar to petroleum derived synthetic plastics like polypropylene (PP) and is completely biodegradable in the environment (Khardenavis *et al*., 2007).

In 1997, Chua et al. stated that the cost of producing PHB bioplastic is ten times higher than that of conventional plastics. Later in 2004, Serafim *et al*. reported that the cost has reduced to nine times higher than the cost of conventional plastics. Up to this day, there have been, and still are, many efforts done by scientists and researchers in order to optimize the production of PHB and reducing the cost. For instance the development of better bacterial strains, and efficient fermentation and recovery systems (Choi *et al*., 1998; Wang and Lee, 1997; Lee, 1996). On the other hand, excess activated sludge from a wastewater treatment plant is used as a source of PHB, and renewable carbon resources derived from agriculture or industrial wastes are used as substrate for PHB accumulation (Suresh Kumar *et al*., 2004; Chua and Yu, 1999; Braunegg *et al*., 1978). By these approaches, the cost on biomass generation can be reduced, apart from volume reduction of waste activated sludge by extracting PHB (Khardenavis *et al*., 2007).

Researches have been done in accordance to selection of efficient PHB producers. Among the PHB producers are *Alcaligenes* sp., *Azotobacter* sp., *Bacillus* sp., *Nocardia* sp., *Pseudomonas* sp., and *Rhizobium* sp. These microorganisms need carbon as their energy source. Plant waste fibers such as banana stem waste have high content of cellulose, which can be used as carbon sources for microorganisms. However, in order to obtain the cellulose, the banana stem waste need to be delignified. In previous studies, several microorganisms have been tested for their ability to degrade lignin on different raw materials. Nonetheless, the aim of this study is to assess the cellulose recovery potential through lignin degradation by bacteria *Bacillus cereus* using banana stem as feedstock

## 1.2 Objectives of Study

Based on the background of this study, the objectives of this study are listed as following:

i. To study the submerged fermentation in anaerobic conditions of banana stem waste using *Bacillus cereus*.
ii. To study the ability of *Bacillus cereus* in delignifying banana stem waste in anaerobic conditions.

## 1.3 Scope of Study

Based on the objectives of this study, the scopes of study are highlighted as follows:

i. Submerged fermentation in anaerobic conditions as the incubation condition for lignin degradation process of banana stem waste using *Bacillus cereus*.
ii. Test the efficiency of lignin degradation of banana stem waste in anaerobic conditions by *Bacillus cereus*.

## 1.4 Problem Statement

Plant waste fibres can be explained as lignocellulosics, which means, resources consist primarily of cellulose, hemicellulose, and lignin. The production of PHB utilizes cellulose. The presence of lignin slows down the microbial attack for the degradation of cellulose. Thus complete degradation does not occur and most of the cellulose remains undigested. To enhance the bioplastic production from such substrate it is necessary to

make cellulose components free from lignin. Biological delignification is a promising process for the preparation of suitable substrate for PHB bioplastic generation. This is where the issue arises. In order to achieve satisfactory delignification, suitable microorganisms have to be used. In the earlier researches, many microorganisms such as actinomycetes, fungi and bacteria have been tested for the ability to delignify lignocellulosics. By using banana stem waste as lignocellulic source; this study aims to discover the ability of *Bacillus cereus* to biologically degrade lignin in banana stem in order to attain the cellulose for PHB production.

## 1.5 Rationale and Significance

The degradation of lignin in banana stem waste by using bacteria *Bacillus cereus* brings excellent justifications. Firstly, lignin needs to be degraded in order to obtain the cellulose which can then be further utilized as a substrate in producing PHB bioplastics. Secondly, as the raw material to be used is waste, it is substantially cheap and widely available here in Malaysia since there are a lot of banana plantations in this country. Moreover, banana stem is known to contain a high content of cellulose. Lastly, if the bacteria strain *Bacillus cereus* is able to degrade lignin, the cost will be significantly fewer than that of using chemicals to degrade lignin, due to the elimination of several steps required to recover cellulose. In addition, microbial degradation by *Bacillus cereus* can reduce the probability of contamination of products (i.e. cellulose) by chemical use, since microbial degradation only involves the activity of enzyme.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Bioplastic and Biodegradation

Recently the term ‘bioplastics’ has almost replaced the term ‘biodegradable plastics’. Bioplastics are now commonly regarded as to be a form of plastics derived from natural resources such as wood (cellulose), vegetable oils, sugar or starch.

Biodegradation is the process by which organic substances are broken down by living organisms. In relation to bioremediation of plastic materials, biodegradation is a process that describes the mineralization of organic structures by microorganisms. These microorganisms convert the bioplastics into carbon dioxide, methane, water and biomass.

To compare the performance of bioplastic and conventional, non-biodegradable plastic, they certainly do not have the same performance characteristics but are fit-for-purpose in a range of specific applications. To review the applications of bioplastic back in the past few years, they are only said to be applied on medical fields due to its high production cost; while conventional plastics dominate all other sectors and fields. Nevertheless, nowadays the applications of bioplastic include biodegradable plastic shopping bags, compostable waste collection bags and compostable or biomass-based

food trays and food service packaging. Their applications in other sectors are currently under development, for instance in the automotive and electronic sectors.

The degradation of biodegradable plastics is due to cell-mediated phenomena (micro-organisms, enzymes, fungi, bacteria). When the degradation is the result of the action of microorganisms in a material and the material is eventually converted to water, carbon dioxide, methane and biomass, the material is considered biodegradable. On the other hand, compostable plastics are degradable due to biological processes that occur during composting and are converted into carbon dioxide, water, and biomass. For both biodegradable and compostable plastics, there are no toxic side effects like toxic residue for water, soil, plants or living organisms. Currently these plastics are based on renewable resources. Though, not all biodegradable materials are compostable.

As for the degradation of bioplastics, microorganisms such as fungi and bacteria can metabolize biodegradable bioplastics. The polymer becomes their source of food and energy. The microorganisms then transform the biodegradable plastic product into carbon dioxide, water and biomass. A certain level of temperature, heat, water and oxygen is required by active microorganisms such fungi and bacteria for effective biodegradation.

Poly β-hydroxy butyrate (PHB), the most studied biodegradable plastics, is a very common and widespread storage material in many micro-organisms. PHB gathers as energy reserve material in many microorganisms such as *Alcaligenes* sp., *Azotobacter* sp., *Bacillus* sp., *Nocardia* sp., *Pseudomonas* sp., and *Rhizobium* sp. In many cases, these microorganisms need carbon as their energy source. Cellulose can be a great carbon source. In order to provide the carbon sources, approaches have been directed towards the usage of nutrient-rich waste. However, usually pretreatment to waste need to be done in order to gain the cellulose content. The way to pretreat depends on the waste type used.

In the present studies banana stem waste is used due to its high cellulose content and availability. Microorganism used to do pretreatment to waste is *Bacillus cereus*. *Bacillus cereus* is to be tested for its ability to degrade lignin content in banana stem, in order to obtain cellulose content inside it.

## 2.2 Raw Material for Carbon Source (Banana Stem Waste)

There are various choices of raw materials to be used in the production of biodegradable plastics, namely corn, starch, potatoes, sugarcane, and even the types of renewable raw materials such as biomass fraction present in waste from households, municipal waste, dairy industry, paper mills, forestry, etc. Some specific types of bioplastics can even be produced directly by certain plants. Others need to go through several processes before PHB bioplastic is formed.

In this study, banana stem waste is chosen as raw material to eventually produce PHB. Banana stem is known to contain a high content of cellulose (cellulosic fibre), which can be the substrate for microorganisms producing PHB. Agricultural activity involving banana generates large amounts of residues, because each plant produces only one bunch of bananas. After harvesting the fruits, banana stem (also known as the bare psuedostems) are cut and usually left in the soil plantation to be used as organic material. It has been estimated that for every 60 kg of banana grown, 200 kg of waste stem is thrown away. In Malaysia alone, the area for banana plantation is estimated to be 34, 000 hectars (Abdul Khalil *et al*., 2006). Therefore, by utilizing these wastes, it is hoped to be a way of disposing the waste instead of forgo them. Moreover using banana stem waste can significantly reduce the cost of PHB production as it is cheap and widely available.

## 2.3 Delignification Process

Plant cell wall material is composed of three important constituents: cellulose, lignin, and hemicellulose. Lignin is a complex polymer of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. This complexity has thus far proven as resistant to detailed biochemical characterization as it is to microbial degradation, which greatly impedes our understanding of its effects.

Nonetheless, some organisms, particularly fungi, have developed the necessary enzymes to break lignin apart. The initial reactions are mediated by extracellular lignin and manganese peroxidases, primarily produced by white-rot fungi (Kirk and Farrell, 1987). Actinomycetes can also decompose lignin, but typically degrade less than 20 percent of the total lignin presents (Crawford, 1986; Basaglia *et al*., 1992).

Lignin degradation is primarily an aerobic process, and in an anaerobic environment lignin can persist for very long periods (Van Soest, 1994). Because lignin is the most recalcitrant component of the plant cell wall, the higher the proportion of lignin the lower the bioavailability of the substrate. The effect of lignin on the bioavailability of other cell wall components is thought to be largely a physical restriction, with lignin molecules reducing the surface area available to enzymatic penetration and activity (Haug, 1993).

## 2.2 Microorganism

### 2.2.1 Nature of *Bacillus cereus*

*Bacillus cereus* is a gram positive, rod-shaped bacterium that could grow optimally at a temperature range of 30 – 37 $^{o}$C with a 250 rpm shaker speed (Valappil *et al.*, 2007). Even though, some strains can grow up to 55 $^{o}$C while others can grow as low as 4 – 5 $^{o}$C. Many strains from dairy products are able to grow at low temperatures. The growth of *B. cereus* is best in the presence of oxygen, even so, they grows well anaerobically.

*Bacillus cereus* is a bacterium that has been known as an agent to food poisoning since 1955 (SCIENCE Magazine, 2004). It is a spore-forming organism that occurs naturally in most food (ESR Ltd., 2001). It causes two types of foodborne illnesses- emetic illness and a diarrhoeal illness. The scientific classification of *Bacillus cereus* is shown in Table 2.1.

**Table 2.1**: Scientific classification of *Bacillus cereus* (Frankland & Frankland 1887)

| | |
|---|---|
| **Kingdom** | Bacteria |
| **Phylum** | Firmicutes |
| **Class** | Bacilli |
| **Order** | Bacillales |
| **Family** | Bacillaceae |
| **Genus** | *Bacillus* |
| **Species** | *cereus* |
| **Binomial name** | *Bacillus cereus* (or *B. cereus*) |

*Bacillus cereus* is a newly characterized strain in the PHB-producing area thus less information regarding its ability to degrade lignin is available elsewhere. There is a research done by Valappil *et al.* (2007) stated that *B. cereus* was found to produce PHB at certain concentration of its dry cell weight, using glucose as the main carbon source. On the other hand, in accordance to research done by Vargas-García *et al*. (2007), 13 strains consist of five bacteria, one actinomycete and seven fungi had been studied for their ability to biodegrade lignocellulose. From the research it has been proven that *Bacillus licheniformis* was able to decrease the concentration of hemicelluloses, apart from showing high lignin degradation activity. Since *Bacillus licheniformis* and *Bacillus cereus* both are of the same genus, it is expected that *B. cereus* also have the ability to degrade lignin in banana stem waste almost as much as *B. licheniformis* did.

# CHAPTER 3

# METHODOLOGY

## 3.1 Introduction

This chapter discusses the materials and procedures for this undergraduate research project. There is four parts of experiment for this research- the revival of bacteria, fermentation analysis, delignification experiment, and delignification analysis. All of these parts are to be discussed further later in this chapter.

## 3.2 Revival of Bacteria

The L-dried specimens of *Bacillus cereus* was purchased from Nite Biological Resource Center (NBRC). The procedures to opening of ampules and revival of this specimen were attached together when purchased.

There were five steps in opening of ampoules and revival of L-dried specimens. First, rehydration fluid and growth medium were prepared as specified in the invoice. Second, the ampule was scored near the middle point of the cotton plug with an ampule cutter. Third, alcohol-dampened gauze was used to disinfect the ampule. Next, a sterile gauze that had not been dampened with alcohol was used to be wrapped around the ampule so as to break it carefully. Lastly, about 0.2 mL of rehydration fluid was added

to the L-dried cells by using a sterile pipette. The cell suspension was well-mixed before it was transferred to agar plate and incubated overnight at 30 $^{o}$C.

### 3.3 Fermentation Analysis

Basically, the fermentation process was done under submerged fermentation and anaerobic conditions by using 250 mL round bottom flask bottles.

Before the delignification experiment could be run, an analysis on fermentation time of *Bacillus cereus* was done in order to figure out the lifetime of this bacterium. After regeneration, the bacteria were inoculated (10% v/v) into liquid suspension and incubated in incubator shaker for 30 hours. Next, they were inoculated again for activation purposes, and incubated in incubator shaker for another 18 hours. Finally the fermentation of *Bacillus cereus* under anaerobic conditions was done by using liquid medium without the banana stem wastes.

Samples were harvested every three hours out of 30 hours fermentation period, followed by every six hours sampling for the rest of fermentation period. Every time a sample was harvested, the absorbance value of sample was recorded. The data obtained from this analysis was used to plot the growth curve of *Bacillus cereus* in anaerobic conditions.